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RESEARCH ARTICLE

Fine-scale population genetic structure in Alaskan Pacific halibut (*Hippoglossus stenolepis*)

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Abstract Pacific halibut collected in the Aleutian Islands, Bering Sea and Gulf of Alaska were used to test the hypothesis of genetic panmixia for this species in Alaskan marine waters. Nine microsatellite loci and sequence data from the mitochondrial (mtDNA) control region were analyzed. Eighteen unique mtDNA haplotypes were found with no evidence of geographic population structure. Using nine microsatellite loci, significant heterogeneity was detected between Aleutian Island Pacific halibut and fish from the other two regions (F_{ST} range = 0.007–0.008). Significant F_{ST} values represent the first genetic evidence of divergent groups of halibut in the central and western Aleutian Archipelago. No significant genetic differences were found between Pacific halibut in the Gulf of Alaska and the Bering Sea leading to questions about factors contributing to separation of Aleutian halibut. Previous studies have reported Aleutian oceanographic conditions at deep inter-island passes leading to ecological discontinuity and unique community structure east and west of Aleutian passes. Aleutian Pacific halibut genetic structure may result from oceanographic transport mechanisms acting as partial barriers to gene flow with fish from other Alaskan waters.

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Introduction

Pacific halibut (*Hippoglossus stenolepis*) are distributed across the North Pacific Ocean from California to the northern Sea of Japan (Allen and Smith 1988; Mecklenburg et al. 2002). Pacific halibut represent an important commercial, sport, and subsistence fishery resource throughout the eastern Pacific Ocean. This flatfish has been fished by indigenous peoples for thousands of years and has sustained intensive commercial harvest for the last century with a rate of ~70 million pounds per year harvested over the last decade (IPHC 1998, 2001; Wilderbuer et al. 2005). Despite the commercial and social importance of this species, little is known about population structure throughout its geographic range. Previous studies have attempted to define population structure for Pacific halibut using allozymes (Tsuyuki et al. 1969; Grant et al. 1984), microsatellite loci (Bentzen et al. 1998; Hauser et al. 2006), parasite load (Blaylock et al. 2003) and tagging experiments (Skud 1977; Seitz et al. 2003; Loher and Seitz 2006b), but only weak evidence has been shown to support discrete populations.

Pacific halibut are large (up to 250 kg) benthic, highly migratory marine fishes that spawn at depth during the winter spawning season (Thompson and Van Cleve 1936; Best 1981). In the eastern Pacific Ocean these fish are managed by the International Pacific Halibut Commission (IPHC) as a single panmictic population or stock (Clark and Hare 2000). This management structure is based primarily on long-term tagging studies over the last 80 years demonstrating significant marine adult migrations (Skud 1977; Kaimmer 2000) and evidence from larval drift

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studies showing northwestern drift of larval halibut from the Gulf of Alaska into the Bering Sea (Skud 1977). However, concerns about local depletion of Pacific halibut in commercial fisheries (Hare 2004) and evidence of migration patterns from recent electronic tagging studies (Seitz 2006) have suggested that marine habitat discontinuities and fidelity to unique spawning locations may support localized population structure.

Population genetic theory predicts that intraspecific divergence occurs over different scales of time and space (Avise et al. 1979, 1987). Geologic and hydrologic barriers that limit gene flow have been well described in freshwater and terrestrial systems, but their impacts on genetic population structure in the ocean remain poorly documented (Palumbi 1994). In addition to absolute barriers to gene flow, population segregation can derive from spatial and environmental conditions that limited dispersal at different life stages (Palumbi 1992, 1994). Vicariance, ecology and physical factors limiting dispersal can play important roles in marine fish population genetic structure (Waters et al. 2000; Ringinos and Nachman 2001; Cowen et al. 2006). Not all individuals within a species behave in a predicted manner when subject to environmental factors leading to genetic segregation (Bernardi et al. 2003). Intraspecific segregation can operate across a broad set of dimensions and robust inference on population structure is not necessarily limited to extremes in distance or time (Brown and Stepien 2008).

Studies have suggested physical oceanographic conditions may have significant impacts on population structure of marine fishes at different geospatial scales (Grant and Bowen 1998; Able et al. 2005; Knutsen et al. 2007; Eckert et al. 2008). Analyses of physical oceanography and bathymetric barriers have been shown to influence population structure of some marine flatfishes (Hemmer-Hansen et al. 2007; Knutsen et al. 2007; Larsen et al. 2007). A recent study of Greenland halibut (*Reinhardtius hippoglossoides*) demonstrated that spawning location, transport depth, and freshwater inflow rates were important factors influencing the settlement and distribution of juvenile Greenland halibut in the north-east Arctic (Ådlandsvik et al. 2004). Spencer (2008) documented that the distribution of several flatfish species in the Bering Sea since 1982 were correlated with movements of the oceanic 'cold pool' (bottom water <2°C). However, genetic analyses of most marine species including flatfishes have shown only weak or limited population genetic substructure across broad geographic areas, suggesting that significant mixing prevents the development or maintenance of genetically differentiated stocks (Vis et al. 1997; Hoarau et al. 2002a, b; Reid et al. 2005; Florin and Höglund 2007).

Genetic studies of Pacific halibut are also limited. Tsuyuki et al. (1969) analyzed a single protein (an iron-binding serum transferrin/hemoglobin) for polymorphisms in Pacific halibut taken from the eastern Bering Sea, northeastern Pacific Ocean and southern British Columbia; no geographic relationship between alleles or allele frequencies was found. Analyses by Grant et al. (1984) using 35 allozyme loci indicated no differentiation between Gulf of Alaska and Bering Sea Pacific halibut samples, but reported heterogeneity between a Japanese sample and the other ocean regions at one allozyme locus (adenine deaminase, *Ada*). Bentzen et al. (1998) developed three microsatellite loci in *H. stenolepis*, but these loci were considered too polymorphic (observed heterozygosity >93%) to detect regional-scale genetic structure for samples taken from Russia, Gulf of Alaska and Washington state. Heterozygosities at the three loci studied by Bentzen et al. (1998) reached or approximated the limiting value of 100% which limited their utility in detecting population subdivision. Pairwise F_{ST} estimates among regions were extremely low (F_{ST} range = 0.0009–0.003) and insignificant; however, significant locus-specific heterogeneity was reported between Russia and Washington samples at two loci ($P = 0.015$ and 0.023) and between Russia and Gulf of Alaska at one locus ($P = 0.008$). These results suggest a weak east–west axis for Pacific halibut population structure in the North Pacific Ocean. Hauser et al. (2006) successfully optimized a suite of 14 microsatellite loci previously described for Atlantic halibut (*Hippoglossus hippoglossus*) for amplification in Pacific halibut and examined allele frequencies from three sites: Newport OR, St. Paul Island in the Bering Sea and Adak Island in the Aleutian Archipelago. Hauser et al. (2006) reported no significant genetic differentiation among the three sites using standard measures. However, permutation tests yielded significant F_{ST} results at the 10% level in pairwise comparisons between the Adak sample and samples from the other two sites.

Linking genetic diversity with highly variable physical forces that help drive population structure is clearly a key to understanding fine-scale genetic population structure in marine fishes (Grant and Bowen 1998; Hemmer-Hansen et al. 2007). Many studies have suggested that population structure of benthic marine fishes is influenced by larval transport dependent on oceanic current systems and wind patterns (Parker 1989; Koutsikopoulos et al. 1991; Nielsen et al. 1998; Stepien 1999; Bailey and Picquelle 2002; Wilderbuer et al. 2002; Knutsen et al. 2007). Benthic species can experience genetic homogenization as a consequence of mixing during the transport of pelagic eggs and larvae despite geographically distinct spawning locations (Hauser et al. 2006; Rooper et al. 2006; Bailey et al. 2008). Well described oceanographic currents flowing from the Gulf of Alaska into the Bering Sea (Stabeno et al. 1999, 2002; Ladd et al. 2005) have been suggested as the

transport mechanism leading to genetic homogenization among Pacific halibut populations in Alaska (Skud 1977; St. Pierre 1989; Hauser et al. 2006).

Data directly linking Pacific halibut biology and oceanography are scarce (see however, Parker 1989; and for other marine flatfishes see Werner et al. 1997 and Bailey et al. 2005). Currently, there are no data for Pacific halibut demonstrating how adults locate spawning grounds, if there is year-to-year fidelity for those locations, or if ocean conditions at these locations contribute to local adaptation by facilitating egg and larval transport and subsequent retention in nursery habitats (see however, Norcross et al. 1999; Seitz et al. 2007). There are still many unanswered questions about the egg stage and larval drift in this species that can spawn at over 500 m depth off the continental shelf with complex ontogenetic requirements to reach nearshore nursery habitats (Norcross and Shaw 1984; Bailey et al. 2008). Egg and larval transport are particularly enigmatic in Pacific halibut where nursery habitats are at such great distance from deep water spawning grounds and larval drift patterns are frequently orthogonal to prevailing surface currents (Bailey et al. 2008).

In this paper we examine fine-scale genetic population structure in adult Pacific halibut collected at three geographically distinct locations in Alaska where adult halibut movements have previously been studied using Pop-up Archival Transmitting (PAT) tags (Seitz et al. 2003, 2007; Loher and Seitz 2006a, b). Coupling physical oceanography with genetic population data allowed us to hypothesize that weak, but significant, population genetic structure described for Pacific halibut in the central and western Aleutian Islands may result from ocean conditions unique to that area.

Materials and methods

Sample collections and DNA extraction

A total of 228 adult Pacific halibut samples were collected during monitoring and tagging cruises in three regions in Alaska (Fig. 1; Table 1). Aleutian Islands samples were collected June–August, 2003 near Atka Island ($N = 27$) and Attu Island ($N = 39$); Bering Sea samples were taken near St. Paul Island in August, 2002 ($N = 57$); and Gulf of Alaska samples were collected from Aialik Bay ($N = 36$), Harris Bay ($N = 53$) and Resurrection Bay ($N = 16$) in March–July, 2001.

Non-lethal samples of caudal fin tissue were removed from each fish and stored in pre-labeled vials containing 100% ethanol. Fin tissue samples were transported to the USGS Alaska Science Center for DNA extraction and genetic analyses using microsatellite and mitochondrial DNA (mtDNA) loci. Genomic DNA was extracted from fin tissue using a Puregene Tissue kit (Minneapolis, MN) following the manufacturer's instructions.

Microsatellite loci development

Three microsatellite loci (*Hst7a*, *Hst14*, and *Hst17*) were developed and optimized in our laboratory using a partial genomic library derived from *H. stenolepis*. Cloning of microsatellite loci was based on the procedure reported in Kandpal et al. (1994). Genomic DNA extracted from *H. stenolepis* was digested with the *Sau3A1* restriction enzyme and size fractioned in a 1.2% agarose gel. Fragments ranging from 400 to 1,500 base pairs (bp) were purified and ligated to *Sau3A* linkers. Linker-ligated

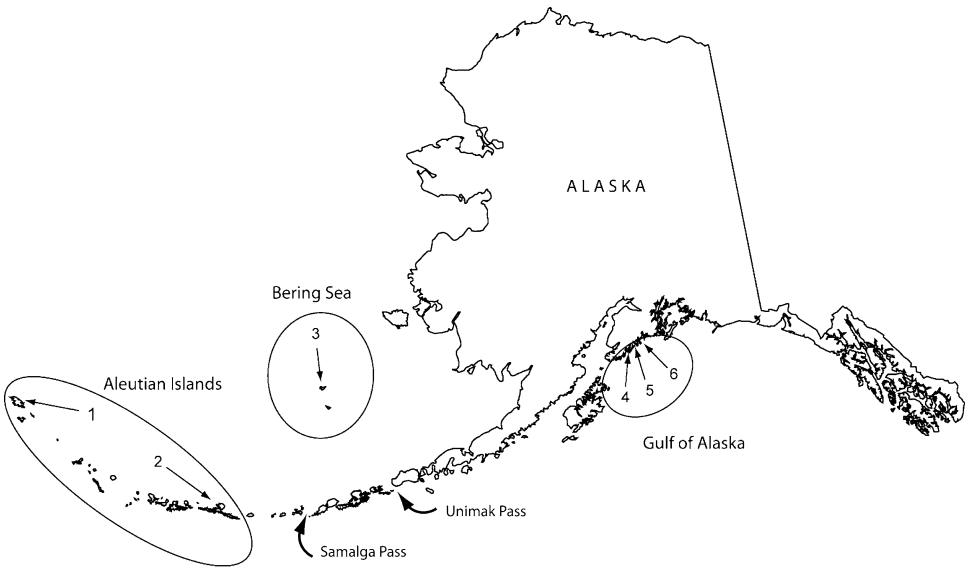

Fig. 1 Map of regional sampling locations for Pacific Halibut collected in this study. Aleutian Island samples—(1) Attu Island ($N = 39$) and (2) Atka Island ($N = 27$); Bering Sea samples—(3) St. Paul Island ($N = 57$); Gulf of Alaska samples—(4) Harris Bay ($N = 53$), (5) Aialik Bay ($N = 36$) and (6) Resurrection Bay ($N = 16$). Samalga and Unimak passes are indicated

Table 1 Regional locations of Alaskan Pacific halibut (*H. stenolepis*) sampled for this study

Region	Population (*N*)	*N*	Average # alleles	Average A_R	Private allelic richness	H_o	H_e
Aleutian Islands	Atka Island (27)	66	13.1	4.42	1.48	0.723	0.683
	Attu Island (39)						
Bering Sea	St. Paul Island (57)	57	12.2	4.46	1.49	0.723	0.688
Gulf of Alaska	Aialik Bay (36)	105	14.4	4.35	1.34	0.708	0.687
	Harris Bay (53)						
	Resurrection Bay (16)						

The number of samples per region (*N*), average number of alleles per locus, allelic richness (A_R), private allelic richness across nine loci, observed heterozygosity (H_o), expected heterozygosity (H_e), and the number of samples taken at the population scale (listed in parentheses) are given for each geographic region

products were amplified using a single oligonucleotide primer based on the linker sequence and amplified products were probed with $(CA)_{12}$ and $(GA)_9$ biotinylated repeats (Operon Technologies, Huntsville, AL). Hybridized fragments were captured and retained on a Vectrex Avidin D matrix (Vector Laboratories, Inc., Burlingame, CA). Fragments enriched with (CA) or (GA) repeats were eluted from the Vectrex Avidin D matrix, ligated into the lacZα gene of the pCR®2.1 cloning vector and transformed into One Shot® INVaF' chemically competent *Escherichia coli* cells (Invitrogen, Carlsbad, CA). Transformed cells were grown on Luria Bertani (LB) agar containing ampicillin (100 mg/ml) and X-gal (5-Bromo-4-Chloro-3-Idoly-β-D-Galactopyranoside; 20 mg/ml).

Positive *E. coli* colonies (white) were individually subcultured in LB-ampicillin broth and cloning vectors pCR®2.1 were isolated using the Wizard Plus ® Miniprep DNA Purification System (Promega, Madison, WI). Isolated pCR®2.1 cloning vectors were bi-directionally sequenced using a SequiTherm EXCEL II DNA Sequencing Kit (Epicentre Biotechnologies, Madison, WI). Sequences were visualized on a LI-COR Model IR2 automated fluorescent DNA sequencer, proofed using AlignIR software (LI-COR, Lincoln, NE) and assessed for microsatellite repeats. Initial primer development used Primer3 (Rozen and Skaletsky 1998). Prospective microsatellite loci were amplified in *H. stenolepis* and assessed for polymorphism. Subsequent primer optimizations (if required) were developed by eye.

Microsatellite loci amplification

Thirty-two microsatellite loci were surveyed for this study, 17 from the published literature on flatfishes and 15 developed in our laboratory from our Pacific halibut partial genomic library (complete list available upon request from author). We selected loci for analyses based on ease of amplification and the competency and reliability of allelic structure on the LI-COR platform. Extensive quality control of results using blind scoring by two independent reviewers was implemented. A subset of samples (>10% of samples) were rerun on new gels to limit probability for genotyping errors. Nine loci were retained in our analyses: six microsatellite loci developed from other flatfish species and three microsatellite loci developed and optimized for this study (Table 2). Microsatellite loci were amplified using the polymerase chain reaction (PCR) in 10 µl volumes containing 25–50 ng of genomic DNA, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 0.01% NP-40, 0.01% Triton X-100, 0.5–1 µM each unlabeled primer, 0.5–1.5 µM labeled tail primer, 2 mM each dNTP and 0.1 U of Promega Taq DNA polymerase (Madison, WI). The PCRs were carried out in MJ Research (BIORAD, Hercules, CA) or MWG thermocyclers (MWG Biotech Inc., High Point, NC) with an initial denaturation time of 2 min at 94°C followed by 40 cycles of 94°C for 15 s, 50°C for 15 s, 72°C for 30 s and a final 30 min elongation step at 72°C. Two loci, *Hst7a* and *Hst14*, required an annealing temperature of 52°C. Primer sequences for the amplification of microsatellite loci developed in this study were: *Hst7a* forward (F) 5′-GC TCAGAGAAAACACACAGCA-3′; *Hst7a* reverse (R) 5′-GCTGTTTGGGGAACTCAATG-3′; *Hst14* (F) 5′- ATGG GAAATGG ACATGGGTA-3′; *Hst14* (R) 5′-GGAG CTGGCAACAAAGACAT-3′; *Hst17* (F) 5′-GCCCTCCT GGTTTGTTGCC-3′; *Hst17* (R) 5′-GTGAGTGGTGGACC GTTGC-3′.

PCR products were separated on 6% polyacrylamide gels using a LI-COR Model IR2 automated fluorescent DNA sequencer (LI-COR). To visualize alleles, each locus' forward primers were synthesized with universal tails on the 5′ ends: *Hhi51*, *Hhi56*, *Hhi59* and *Hst17* were synthesized with M13 (F) tails; *Hhi3*, *Vva13* and *Vmo17* with M13 (R) tails; and *Hst7a* and *Hst14* with T7 tails. Fluorescently labeled primers complementary to the tailed sequences were added to the PCR mixture to visualize alleles. Allele sizes for specific samples at each locus were determined relative to the M13 phage single nucleotide ladder and these samples were used as internal size standards to accurately score alleles in subsequent gels. Gene

Table 2 Characteristics of nine microsatellite loci used in this study

Locus	Source	Cloned repeat motif	Developed in	Range (bp)	N_A	A_R	Accession number
Hhi3	Coughlan et al. (2000)	$(CA)_{32}$	*Hippoglossus hippoglossus*	147–245	40	7.76	AJ270780
Hhi51	Coughlan et al. (2000)	$(TG)_8AG(TG)_5$	*Hippoglossus hippoglossus*	96–172	28	4.23	AJ270781
Hhi56	Coughlan et al. (2000)	$(GT)_2AT(GT)_{12}$	*Hippoglossus hippoglossus*	181–223	11	3.17	AJ270785
Hhi59	Coughlan et al. (2000)	$(CT)_2(GT)_{12}$	*Hippoglossus hippoglossus*	137–155	10	3.52	AJ270787
Hst7a	This study	$(CT)_9TTGTTT(CT)_7$	*Hippoglossus stenolepis*	169–209	14	3.91	DQ979362
Hst14	This study	$(CA)_4TACTGTA(CA)_{15}$	*Hippoglossus stenolepis*	163–213	26	5.82	DQ979363
Hst17	This study	$(GT)_{13}$	*Hippoglossus stenolepis*	142–178	16	5.51	DQ979364
Vmo17	Ortega-Villaizán Romo et al. (2003)	$(GA)_{29}$	*Verasper moseri*	166–176	3	4.26	AB110623
Vva13	Ortega-Villaizán Romo et al. (2003)	$(CA)_{25}$	*Verasper variegatus*	101–125	13	1.50	AB110629

Allelic size ranges (*bp* base pairs); number of alleles (N_A); allelic richness (A_R) and the GENBANK accession number are given by locus

ImagIR v4.05 software was used to assign allele scores (LI-COR). Approximately 10% of all samples were independently amplified and scored to verify allele sizes across all loci for quality control. MICROCHECKER (van Oosterhout et al. 2004) was used to test for null alleles at all nine loci.

Mitochondrial DNA

A 254 bp fragment of the mtDNA control region in *H. stenolepis* was amplified using primers developed for European plaice, *Pleuronectes platessa* L. (Hoarau et al. 2004). The forward primer, DLF (5′-CCACCTCTAACTC CCAAA GC-3′), is located within the 3′ end of the tRNA proline gene, and the reverse primer, DLR (5′-TGAAGGG ATTTTGAGTCTTGG-3′) occurs within the control region. M13 (F) and M13 (R) tails were synthesized to the 5′ ends of DLF and DLR primers, respectively, and fluorescently labeled primers complementary to the tailed sequences were incorporated into the sequencing reaction mixture to illuminate the products. Bi-directional sequencing of the Pacific halibut mtDNA control region was done using the SequiTherm EXCEL II DNA Sequencing Kit (Epicentre Biotechnologies). Sequences were separated in 5.5% polyacrylamide gels, visualized on a LI-COR Model IR2 automated fluorescent DNA sequencer and aligned using AlignIR software (LI-COR).

Population genetic statistics

Allelic size ranges and the total number of alleles (N_A) was calculated for each locus with Microsatellite Toolkit v2.0 (Park 2001). Allelic richness (A_R) and the average number of private alleles across all nine loci were calculated using HP-RARE v1.0 (Kalinowski 2005). Observed and expected heterozygosity by locus (H_o and H_e, respectively) were calculated for each region using ARLEQUIN v3.01 (Excoffier et al. 2005). Probability tests for Hardy–Weinberg equilibrium (HWE) were done for all regions combined and independently for each region by locus and tests for linkage disequilibrium between loci were performed using GENEPOP v3.4 (Raymond and Rousset 1997). Weir and Cockerham's (1984) locus-specific measure of departure from HWE (F_{IS}) was generated to detect significant heterozygosity excess (negative F_{IS} values) or deficit (positive F_{IS} values) using GENEPOP v3.4. Genetic differentiations between regions were tested with pairwise F_{ST} comparisons using ARLEQUIN v3.01. A Bonferroni correction based on the number of loci ($P = 0.05/9 = 0.0056$) was used to evaluate significance in all tests (Rice 1989). Fishers exact test comparing population pairs across all loci were performed using GENEPOP. Using the computer program SAMOVA 1.0 (http://web.unife.it/progetti/genetica/Isabelle/samova.html) probabilities of partitions (barriers) of geographically adjacent sampling areas were analyzed based on genotypic data for $K = 2$ and $K = 3$ (Dupanloup et al. 2002). Multiloci estimates of population differentiation following standard ANOVA analogues (Weir and Cockerham 1984; Michalakis and Excoffier 1996) were performed using GENEPOP. Estimates of effective population size for Pacific halibut by region were calculated using LDNe (Walpes and Do 2008).

Mitochondrial DNA analysis

The *H. stenolepis* mtDNA sequence was confirmed by alignment with the published sequence for the mtDNA control region of the Japanese barfin flounder (*Verasper moseri*) also in the Family Pleuronectidae (GENBANK Accession #AB207249; Ortega-Villaizán Romo et al. 2006). Standard diversity indices (number of haplotypes, haplotype diversity and nucleotide diversity) were assessed by region and regional mtDNA haplotype frequencies were analyzed using ARLEQUIN v3.01. AMOVA analysis of haplotype diversity and Tajima's D tests for selective neutrality were performed using ARLEQUIN v3.01. A

haplotype minimum spanning tree was generated using TCS v1.21 (Clement et al. 2000).

Results

Microsatellites

We found little difference in the average numbers of alleles per locus and average allelic richness among halibut from the three geographic regions (Table 1). Average private allelic richness (Kalinowski 2004) was also similar across the regions (Table 1). Allelic size ranges (base pairs), the total number of alleles, allelic richness and GENBANK accession numbers for loci evaluated in this study are reported in Table 2. Evidence of linkage disequilibrium was found between *Hhi51* and *Hhi59* ($\chi^2 = 32.56$; $P < 0.000$), and between loci *Hhi51* and *Hst14* ($\chi^2 =$ infinity; $P < 0.000$) for all samples combined. Coughlan et al. (2000) previously reported potential linkage disequilibrium for other Atlantic halibut loci with *Hhi51*. *Hst14* was the only locus identified by MICROCHECKER to have a potential null allele due to homozygous-excess (homozygotes observed = 48; expected = 32). Allelic size distribution for this locus was large (163–213 bp), but there was no evidence for large allele drop out based on MICROCHECKER analyses. All nine loci were in HWE for the three regions combined. The Aleutian Islands sample was significantly out of HWE at *Hst14* due to heterozygote deficit ($F_{IS} = 0.118$, $P = 0.003$) and at *Hhi56* due to heterozygote excess ($F_{IS} = -0.345$, $P = 0.000$). The Bering Sea and Gulf of Alaska samples were both out of HWE at *Hhi59* due to heterozygote excess ($F_{IS} = -0.377$ and -0.288, respectively).

Significant genetic differentiation based on F_{ST} pairwise comparisons was found between the Aleutian Islands and the Bering Sea ($F_{ST} = 0.0083$) and Gulf of Alaska ($F_{ST} = 0.0078$) Pacific halibut collections (Table 3). No significant genetic differentiation was detected between the Gulf of Alaska and the Bering Sea halibut samples ($F_{ST} = -0.0021$). Running F_{ST} analyses on our sample locations collections without loci lacking HWE at the population level did not significantly change our results. F_{ST} analyses with dropped loci supported significant allelic differences between the Aleutian Islands and the Gulf of Alaska ($F_{ST} = 0.0075$) and the Bering Sea ($F_{ST} = 0.0091$). No significant differences in allelic frequencies were found in these analyses between the Gulf of Alaska and from the Bering Sea ($F_{ST} = -0.0013$).

Fisher's exact tests for population pairs across all loci gave similar non-significant results between the Gulf of Alaska and Bering Sea halibut populations ($\chi^2 = 25.07$; $df = 18$; $P = 0.123$). Exact tests also supported significant differentiation between the Aleutian and Gulf of Alaska populations ($\chi^2 =$ infinity; $df = 18$; $P =$ highly significant) and between the Aleutian and Bering Sea populations ($\chi^2 =$ infinity; $df = 18$; $P =$ highly significant). AMOVA analysis partitioned 99.5% of microsatellite allelic variation within populations. Only 0.4% of the variance was found among locations. LDNe estimates of effective population size were Gulf of Alaska $N_e = 324$; Bering Sea $N_e = 280$; Aleutian Islands $N_e = 273$. Upper 95% confidence limits for all three N_e estimates were infinite under both parametric and jackknife simulations.

Table 3 Pairwise F_{ST} values and *P*-values (in parentheses) for regional comparisons of Pacific halibut based on nine loci

	Aleutian Islands	Bering Sea
Bering Sea	**0.0082 (0.001)**	
Gulf of Alaska	**0.0069 (0.000)**	−0.0016 (0.797)

Significant differences between regions are indicated in bold

Mitochondrial DNA

Eighteen haplotypes were detected with 13 variable nucleotide positions (Table 4). The mtDNA control region was sequenced from $N = 95$ samples taken at random representing 35–44% of all samples from each collection location within a region. *H. stenolepis* (HST) haplotype frequencies by region are reported in Table 5. The Gulf of Alaska sample contained eight unique mtDNA haplotypes: HST3 ($N = 3$); HST4 ($N = 2$); HST5 ($N = 4$); HST7 ($N = 1$); Hst13 ($N = 1$); HST14 ($N = 1$); HST15 ($N = 1$); HST16 ($N = 1$). Two halibut with unique haplotypes were found in the Aleutians (HST10 and HST18) and one unique haplotype occurred in one Bering Sea sample (HST17). Genetic diversity was higher for mtDNA data (average $h = 0.818$; Table 6) compared to microsatellite data (average $H_o = 0.718$; average $H_e = 0.686$), but was similar among the three geographic regions. Analyses of groups of geographically adjacent samples using SAMOVA where $K = 2$, partitioned halibut populations east and west (Group1 = Bering Sea and Gulf of Alaska; Group2 = Aleutians; $F_{CT} = 0.382$). However, SAMOVA analysis when $K = 3$ with each population representing one geographic group gave similar results $F_{CT} = 0.367$). Tajima's *D* statistic (Aleutians $D_t = -00.08$; Bering Sea $D_t = -0.13$; Gulf of Alaska $D_t = -0.25$) were not significantly different from zero ($P > 0.05$ in all cases) and departures from neutrality under the Infinite Allele Model (IAM) could not be differentiated from demographic factors. AMOVA results showed that only 1.45% of all haplotype variation was found among the three regions; 98.5%

Table 4 Mitochondrial control region variable nucleotide positions for 18 *H. stenolepis* (HST) haplotypes

Haplotype	Variable nucleotide positions													Gen Bank accession number
	1	15	75	79	86	110	161	169	171	172	173	189	193	
HST 1	G	C	A	G	T	C	A	A	A	A	T	A	G	EU840228
HST 2	.	.	.	A	.	.	.	.	.	.	.	.	.	EU840229
HST 3	.	.	.	.	.	.	.	.	G	.	.	.	A	EU840230
HST 4	.	.	.	.	.	.	G	.	G	.	.	.	A	EU840231
HST 5	.	T	.	A	.	.	.	.	G	.	.	.	A	EU840232
HST 6	.	.	.	A	.	.	.	.	G	.	.	.	A	EU840233
HST 7	.	.	.	A	.	.	.	C	.	.	.	.	A	EU840234
HST 8	.	.	.	A	.	.	.	.	.	.	.	.	A	EU840235
HST 9	A	.	.	A	.	.	.	.	.	.	.	.	.	EU840236
HST 10	.	.	G	A	.	.	.	.	G	.	.	.	A	EU840237
HST 11	.	.	.	A	.	.	.	.	.	.	C	.	.	EU840238
HST 12	.	.	.	A	.	.	.	.	.	.	.	G	A	EU840239
HST 13	.	.	.	.	.	.	.	.	.	G	.	.	.	EU840240
HST 14	.	.	.	.	.	.	.	G	G	.	.	.	A	EU840241
HST 15	.	.	.	A	C	.	.	.	.	.	.	G	A	EU840242
HST 16	.	.	.	A	.	.	.	G	G	.	.	.	A	EU840243
HST 17	.	.	.	A	.	T	.	.	G	.	.	.	A	EU840244
HST 18	.	.	.	.	C	.	.	.	G	.	.	.	A	EU840245

Base pair numbering scheme was determined relative to nucleotide position 1 of the control region of *Verasper moseri* (Accession No. AB207249; Ortega-Villaizán Romo et al. 2006)

Table 5 Pacific halibut mtDNA haplotype frequencies

	Haplotype																		*N*
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
Aleutian Islands	6	7	0	0	0	7	0	2	3	1	1	0	0	0	0	0	0	1	28
Bering Sea	8	4	0	0	0	3	0	2	0	0	1	1	0	0	0	0	1	0	20
Gulf of Alaska	15	9	3	2	4	4	1	2	2	0	0	1	1	1	1	1	0	0	47
Total	29	20	3	2	4	14	1	6	5	1	2	2	1	1	1	1	1	1	95

The total number of haplotypes sequenced (*N*) by region and the total number of each haplotype across regions are given

Table 6 Mitochondrial diversity statistics by region for Pacific halibut haplotypes

Region	N_h	*h*	π
Aleutian Islands	8	0.8095	0.0068
Bering Sea	7	0.8000	0.0064
Gulf of Alaska	14	0.8446	0.0073

N_h number of haplotypes; *h* haplotype diversity; π nucleotide diversity

was found within regions. Haplotype frequencies and the haplotype minimum spanning tree also revealed no significant geographic differentiation among the three regions (Table 6; Fig. 2).

Discussion

Genetic diversity in Pacific halibut collected from three geographically distant locations in Alaska was reported in this study. These studies demonstrated weak but significant east to west differentiation of halibut in Alaskan waters. Microsatellite loci revealed statistically significant differences in allelic frequencies for Pacific halibut collected off Attu (western Aleutians) and Atka (central Aleutians) islands when compared to fish from the two proximate Alaskan shelf regions (Gulf of Alaska and Bering Sea). However, no significant geographic differentiation was observed in mtDNA haplotypes, suggesting female lineages in Pacific halibut are mixed throughout Alaskan waters.

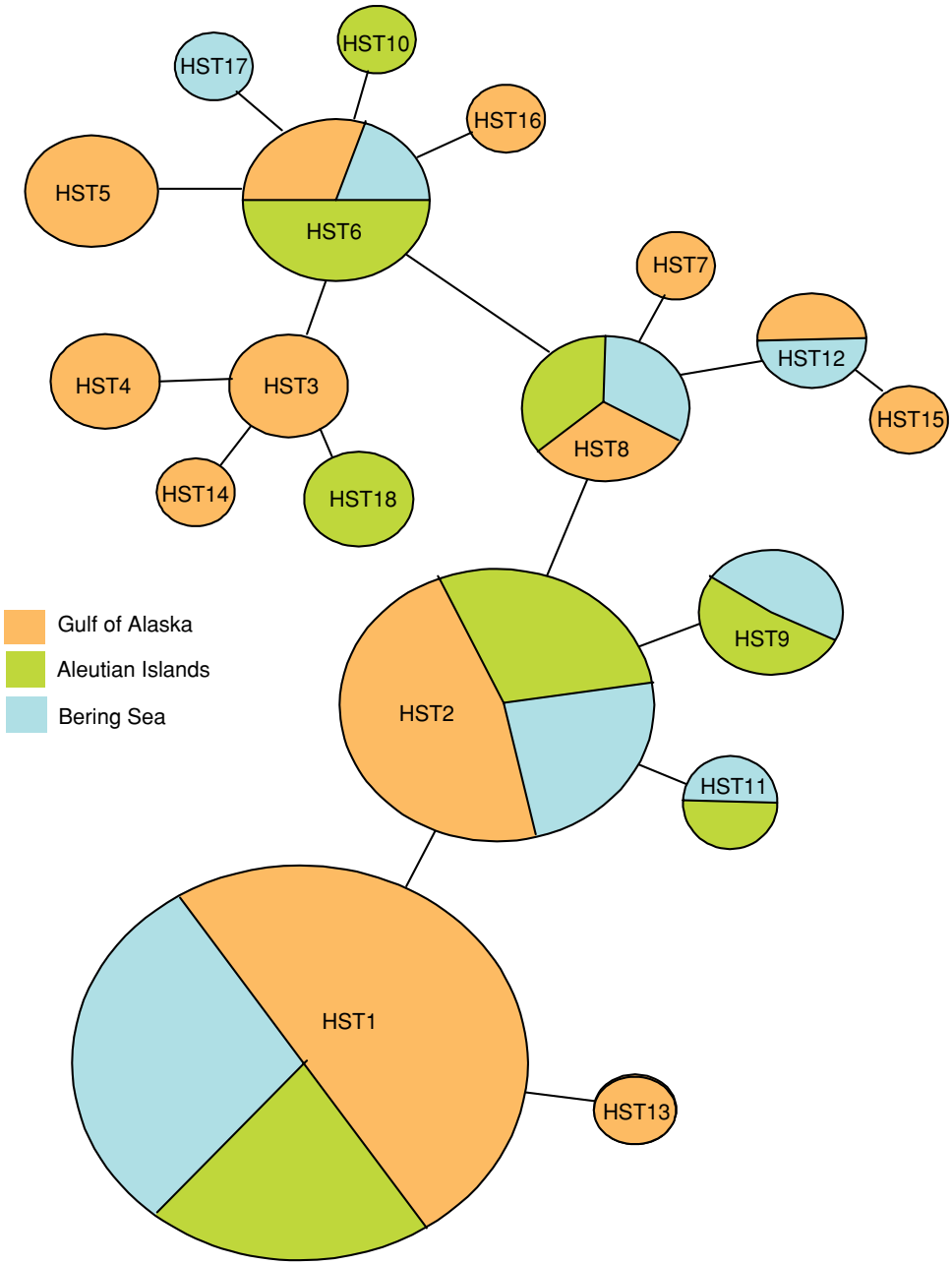

Fig. 2 Minimum spanning mtDNA haplotype network tree. *Sizes of circles* are proportional to frequencies of haplotypes

Genetic diversity was slightly higher for mtDNA sequence data (average $h = 0.818$) compared to microsatellite data (average $H_o = 0.718$), but the scale of genotypic diversity was similar among fishes from the three geographic regions at both molecular markers. Other flatfish studies have shown higher mtDNA diversity compared to diversity found in microsatellite loci (Fujii and Nishida 1997; Hoarau et al. 2004). In contrast to our findings, Hoarau et al. (2004) found reduced genotype diversity in plaice populations from off-shelf habitats (such as Aleutian Island populations) when compared to those living in ocean shelf habitats (such as the Gulf of Alaska and Bering Sea in this study). The theoretical effect of effective size and dispersal ability on genetic population structure has been discussed in the literature (Bohonak 1999; Nunney 1999; Eckert et al. 2008). High effective population size and dispersal ability lead to genetic homogenization across large areas open to dispersal, whereas smaller effective size and greater geographic isolation lead to genetic differentiation. Estimates of effective population size for Pacific halibut across the three regions based on LDNe were similar. However, lack of precision in these estimates which all had infinite upper confidence limits was problematic. Waples (2006) suggested that N_e results with infinite confidence limits may not accurately represent effective size due to noncompliance with model

expectations. Our halibut collections were relatively small for each locality and these data may not be robust under assumptions used by LDNe. True effective size in Alaskan Pacific halibut remains unknown as dispersal patterns, spawning locations and breeding structure in this long-lived species are poorly studied.

Common mtDNA haplotypes were present in Alaskan Pacific halibut across all regions and there was no statistical support for independent population structure at this locus. Previous genetic studies of marine flatfish have been unable to document significant geographic population structure using mtDNA (Fujii and Nishida 1997; Hoarau et al. 2004). The genealogical structure of mtDNA depends on deep evolutionary history in female lineages and can be highly variable in marine organisms suggesting well-mixed populations (Irwin 2002; Wilkins 2004). This appears to be the case in Pacific halibut in Alaska. Subsequent investigations of many marine fishes lacking biogeographic subdivision for mtDNA have revealed local population structure using microsatellite loci (Bentzen et al. 1996; O'Connell et al. 1998; Lundy et al. 1999; Shaw et al. 1999; Mclean and Taylor 2001; Ruzzant et al. 2006). In one case (Aleutian Pacific halibut), our analyses follow this trend of increased resolution with microsatellite loci.

Deviations from HWE for microsatellite loci are often poorly reported in the published literature (Salanti et al. 2005). Despite conformance with HWE at all loci when all sample locations were combined, we reported significant deviations at the population level for three loci (*Hst14*, *Hhi56* and *Hhi59*). Repeated genotyping and quality control checks for these loci showed no genotyping errors as described in Cox and Kraft (2006). HWE is an approximation and specific assumptions built into this test are rarely met in natural populations (Salanti et al. 2005). Clearly, halibut populations in this study were relatively small and probably do not conform to the "infinite population" assumption required for this test. Samples of marine fishes are difficult to obtain from the western and central Aleutian Islands where there are few villages, no commercial fisheries, and limited access. Removal of loci with significant HWE deviation at the population level did not significantly change our primary F_{st} results for population structure.

Microsatellite analyses did not show significant genetic differentiation between the two Alaskan shelf populations of Pacific halibut (Gulf of Alaska and the Bering Sea). Congruent results from two molecular markers supported a relatively high level of gene flow between these geographically distant areas for Pacific halibut. Molecular studies of other flatfishes have reported a lack of population structure in fish with high dispersal ranges at large oceanographic scales (Mork and Haug 1983; Grant et al. 1984; Haug and Fevolden 1986; Vis et al. 1997; Reid et al. 2005; Hauser et al. 2006; Florin and Höglund 2007). Direct or proximate mechanisms leading to this broad-scale geographic mixing for Pacific halibut in Alaska remain speculative. Conventional tagging studies demonstrated movement of age 1–6 year old Pacific halibut dispersing from the Bering Sea to the Gulf of Alaska (Skud 1977). Gene flow facilitated by current exchange and transport of younger life stages as suggested by Skud (1977) may be sufficient to effectively mix these halibut populations genetically. However, these conventional tagging studies have been based on tag recoveries from fish collected in their summer feeding habitats and did not record spawning locations, the point of genetic exchange. Winter spawning locations for Pacific halibut off the shelf in the Gulf of Alaska have been previously described (St. Pierre 1984; IPHC 2001, 1998) and recently confirmed (Seitz et al. 2005; Loher and Seitz 2008), but putative spawning locations in the Bering Sea have not been well documented. A recent study using satellite pop-up (PAT) tags on adult halibut from the Gulf of Alaska and the Bering Sea provided no evidence of movement of adults during spawning migrations between these two areas (Seitz et al. 2003, 2007; Loher and Seitz 2006a), although one PAT tag from a halibut tagged near St. Paul Island in the Bering Sea popped off during the spawning season near Unimak Pass.

Oceanographic studies have shown that a considerable portion of the flow from the Alaska Coastal Current moves northeastward into the Aleutian North Slope Current through the relatively shallow Unimak Pass (max depth = 160 m; Stabeno et al. 2002). Several other passes are known to carry currents between the Gulf of Alaska and the Bering Sea (Stabeno et al. 1999, 2004, 2005; Hunt and Stabeno 2005; Ladd et al. 2005). This exchange of currents greatly influences the marine environment for marine birds and mammals, fish stocks and shellfish in the eastern Aleutian Archipelago and the southeast Bering Sea (Ladd et al. 2005). PAT tagging results covered movements from a small number of fish ($N = 9$) and it is possible that these results may not remain consistent with a larger sample size. We cannot rule out the possibility that Pacific halibut move through these passes at some life stage contributing to gene flow between the two locations.

Significant population structure at smaller geographic scales (individual spawning grounds or bay populations) has been reported for other flatfishes: Atlantic halibut (Haug and Fevolden 1986; Foss et al. 1998), plaice (Hoarau et al. 2002a, b), turbot (*Scophthalmus maximus*; Bouza et al. 2002), and Japanese flounder (*Paralichthys olivaceus*; Sekino et al. 2003). In this study, microsatellite loci revealed statistically significant differences in allelic frequencies for Pacific halibut collected from the western and central Aleutian Islands when compared to fish from the two proximate Alaskan shelf regions (Gulf of Alaska and

Bering Sea). The scale of this molecular differentiation, however, was not large (relatively low F_{ST} values) and inference drawn from these analyses should be considered preliminary. Additional sampling of Pacific halibut along the Aleutian Archipelago will help resolve questions surrounding population independence at different scales and identify potential barriers to gene flow.

It is important to consider mechanisms that may be contributing to population structure in Aleutian Pacific halibut. Recent literature has suggested that unique oceanic characteristics may play a role in fine-scale genetic population structure in marine fishes (Logerwell et al. 2007; Bailey et al. 2008; Selkoe et al. 2008; Cunningham et al. 2009; Galarza et al. 2009) and other marine organisms (Thornhill et al. 2008; Wilson et al. 2009). Well documented oceanic characteristics with the potential to impact Aleutian Pacific halibut include the ocean passes mentioned above and the powerful currents that flow within them. The central and western Aleutian Islands rise steeply from the sea bed with little or no shelf and are separated by relatively deep passes with powerful tidal currents. Water from the oceanic western boundary current, the Alaska Stream, flows through several deep passes between the islands in the central and western Aleutian Archipelago (Ladd et al 2005; Bailey et al. 2008). Many local eddies and gyres surround these islands (Ladd et al. 2005; Hunt and Stabeno 2005; Stabeno et al. 2005). Oceanic conditions have been shown to be important transport mechanisms for micronutrients (Stabeno et al. 2002, 2005; Mordy et al. 2005) and zooplankton in this area (Coyle et al. 1998; Coyle 2005), potentially contributing to larval retention mechanisms. Cunningham et al. (2009) recently presented evidence that gene flow may be restricted in Pacific cod (*Gadus macrocephalus*) where deep-water barriers, such as underwater canyons or swift currents limit adult dispersal. Samalga Pass marks a well-described ecological division of Aleutian waters with strong discontinuity in cold-water corals, zooplankton, other fishes, marine mammals and foraging seabirds, including a step change in species composition (Hunt and Stabeno 2005). If spawning takes place locally, documented oceanic divides, clockwise current circulation (see Ladd et al. 2005, Fig. 6), eddies, and gyres found around islands in the central and western Aleutians may also contribute to retention for halibut eggs/larvae.

Seasonal migrations of Pacific halibut in the Aleutian Islands have only recently been studied (Loher and Seitz 2006b). Loher and Seitz (2006b) PAT tagged 25 adult Pacific halibut in the western Aleutian Archipelago in 2004. Tags were programmed to release during the winter spawning season. These fish did not cross local oceanic passes separating the islands from other shelf or island habitats (Amukta Pass to the east of Atka, Amchitka pass between Atka and Attu, and Near Pass to the west of Attu). Mean dispersal distance of tagged Pacific halibut indicated that fish from the Aleutian Islands move very little compared to those tagged in the Gulf of Alaska and the Bering Sea. On average, Pacific halibut from the Aleutians moved less than 45 km, remaining in local waters throughout the spawning season (Seitz 2006). Limited dispersal patterns may be contributing to the genetic population structure we found for Pacific halibut in the central and western Aleutian Islands.

Because sampling has been sporadic and often not directed specifically to Pacific halibut, we know very little about Alaskan Pacific halibut life histories in the Aleutians. Bathymetric evidence (Ladd et al. 2005; Stabeno et al. 2005; Bailey et al. 2008), recent tagging studies (Loher and Seitz 2006b) and genetics (this study) suggest that one or more passes west of Unimak Pass may represent partial barrier(s) to gene exchange for Pacific halibut in the central and western Aleutian Islands. Boundaries to other marine species distribution are associated with some of these passes (Grant et al. 1983; Logerwell et al. 2007), so it does not seem improbable that the Aleutian deep-water passes may affect population connectivity in Pacific halibut as well.

In summary, behavior and ecology linked to dispersal have been shown to have significant effects on the population dynamics of marine fishes (Botsford et al. 2008). For flat fishes, larval-stage dispersal can be substantial and over large distances (Norcross and Shaw 1984). Genetic population structure may link the dynamics of populations on the scale of that dispersal distance. Adult migrations for spawning in highly migratory marine fishes like Pacific halibut, clearly effect genetic structure. In long-lived Pacific halibut we still have not documented individual reproductive success and fidelity to spawning locations that would be necessary to generate highly rigorous genetic population structure. However, genetic results presented here suggest that dispersal distance and exchange of water masses north and south of relatively shallow oceanic passes, may facilitate genetic exchange and gene flow between Pacific halibut in the Gulf of Alaska and the Bering Sea. Different oceanic conditions associated with deep ocean passes flowing through the steep terrain of the Aleutian Islands potentially contribute to an east/west segregation of Pacific halibut in the central and western Aleutian Islands where unique bathymetry and hydrology may provide sufficient local conditions for Pacific halibut of different life stages.

Intriguing but inconclusive genetic results have been reported comparing Pacific halibut from Japan (Grant et al. 1984) and Russia (Bentzen et al. 1998) with populations in Alaska. A Japanese tagging study of yellowfin sole (*Limanda aspera*) indicated that populations in the western

Bering Sea (west of St. Paul Island and Unimak Pass) remain separate from eastern Bering Sea populations throughout the year (Grant et al. 1983). Inference gained from these studies suggests further consideration is needed for Pacific halibut phylogeography in Asian waters and genetic relationships between those populations and fish living in the central and western Aleutian Islands. More extensive sampling, both biological and oceanographic, could provide additional insight on fine-scale population genetic structure of Pacific halibut and this species' dependence on marine conditions and ocean mechanisms for dispersal and recruitment.

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